Anim. Reprod., v.12, n.3, p.418-427, Jul./Sept. 2015

What is new in the cryopreservation of embryos?

C. Almiñana[1,3], C. Cuello[2]

[1]UMR85, Physiologie de la Reproduction et des Comportements, INRA, Nouzilly, France.
[2]Department of Animal Medicine and Surgery, Faculty of Veterinary, University of Murcia, Campus de Espinardo, Murcia. Spain

Abstract

Embryo cryopreservation represents a pivotal tool for the long-term storage and exchange of valuable genetic resources of livestock and endangered species. The innumerable applications of embryo cryopreservation in human medicine, animal production, as well as in other embryo biotechnologies for research purposes are calling for standardized protocols that can be used in these different fields. This review will provide the reader with a brief outline to "the classics" of embryo cryopreservation procedures in farm animals and with a deeper insight into "the new trends". Moreover, the cryopreservation effects on the embryo will be revised; from the easily visible cellular damage to the damage at the transcriptomic, proteomic and lipidomic level, and fresh attention will be given to the epigenetic effects of this technology. Finally, we will go through personal considerations to take into account when embryo cryopreservation is used: how to select the best embryos for cryopreservation, the eternal question for how long can we store the cryopreserved embryos, the fact that size and fat matter when it comes to embryo cryopreservation and ultimately, our suggestion about designing cryopreservation protocols "*à la carte*" attending the needs of each type of embryo.

Keywords: cryopreservation, embryo, livestock animals, slow freezing, vitrification.

Introduction

Embryo cryopreservation has been a very useful tool for embryology since the first successful cryopreservation of mouse embryos in 1972 (Whittingham *et al*., 1972). This technology is the best method for the long-term preservation of valuable genetic resources from experimental and livestock animals. The use of cryopreservation is also essential for the widespread use of embryo transfer, which allows the exchange of genetics with reduced transportation cost, avoiding animal welfare problems and with a minimal risk of disease transmission. At present, millions of offspring have been born from cryopreserved embryos of more than 40 mammalian species (Saragusty and Arav, 2011).

The improvement of freezing protocols and the development of the vitrification technique have led to great advances in embryo cryopreservation over the last thirty years. Comparing current results with those obtained when the first freezing systems became available, it is evident that we are on the right path. Cryobiologist and reproductive biologists have provided with a better understanding of the physical principals of cryopreservation techniques (Liu *et al*., 2012) and their short- and long-term biological effects on the embryo. Today embryo cryopreservation is routinely used in bovine commercial embryo transfer (ET) programs. According to the 23rd annual report of International Embryo Transfer Society (IETS) of the data collected during 2014 for embryo transfer (ET) activity 2013, almost 60% of the transfers of in vivo derived bovine embryos were performed with cryopreserved embryos with high variations between countries (IETS, 2014). For other domestic animal species (small ruminants, pig, and equine) little is known, mainly due to the lack of reporting activity for these species. In addition, the difficulty and high cost of obtaining large numbers of embryos in these species has limited the number of cryopreservation studies performing ET. However, it is believed that ET activity of cryopreserved embryos is increasing, mainly in pigs.

To date, the absence of a perfect universal protocol and the low survival and farrowing rates obtained using slow freezing in some mammalian species (reviewed in Vajta, 2013) represent the major hurdles for a more widespread use of embryo cryopreservation.

In this review we will present the latest advancements achieved in embryo cryopreservation and some of the big challenges that cryobiologists and reproductive biologists need to overcome in the next years. Our purpose is to give some hints that can serve researchers as a guide for optimizing embryo cryopreservation protocols that can be routinely used in a wide range of species.

Strategies and their principles: slow freezing and vitrification

Two basic strategies have ruled the embryo cryopreservation field: the traditional slow freezing, also referred as conventional "equilibrium freezing" or "controlled slow freezing" and vitrification. In the

[3]Corresponding author: calminana@tours.inra.fr
Phone: +33(2)4742-7508
Received: May18, 2015
Accepted: July 1, 2015

present review we briefly mention the basic principles of these two strategies, since further detailed reviews on these systems can be found elsewhere (Leibo and Songsasen, 2002; Mazur, 2004; Fahy and Rall, 2007).

In the slow freezing process, the embryo is placed in a hypertonic solution containing 1-1.5 mol/L low molecular weight permeable cryoprotectants to facilitate partial embryo dehydration and therefore to avoid intracellular ice crystal formation during cryopreservation. Embryos are slowly cooled (0.2-2.0ºC/min) using a programmable freezer to sub-zero temperatures (-30 to -70ºC), and then plugged intro liquid nitrogen (-196ºC). In this procedure, embryos reach osmotic equilibrium with the cryoprotectants (CPAs) solution before freezing (Palasz and Mapletoft, 1996; Youngs *et al*., 2011). The slow freezing procedure has proven effective for mice, cattle and human embryos. However, for those embryos more sensitive to chilling injury, such as the pig embryo, *in vitro* produced or early stage embryos the slow freezing is not efficient.

Vitrification is defined as the solidification of a water-based solution forming a glass-like amorphous vitreous state without ice crystal formation. Vitrification can be achieved by using high cooling rates and high concentration of CPAs (Fahy *et al*., 1984). Embryo vitrification that was first reported by Rall and Fahy (1985) has two main advantages compared to slow freezing; it eliminates ice formation and reduces chilling injury (reviewed in Kasai and Mukaida, 2004). Vitrification is more effective, much quicker and simpler than controlled slow freezing. Since it does not require computerized equipment, it can be done even under field conditions. However, it requires highly trained personnel for manipulating embryos in small volumes and in short equilibration times. In the vitrification process, the embryo is exposed first to a low CPAs concentration solution (1-1.5 mol/L) and then to a much more concentrated solution (4-8 mol/L). Vitrification relies especially on 2 aspects that are closely linked: 1) A very high cooling rate (around 20000 ºC/min or higher), which is achieved by plunging the sample in liquid nitrogen (-196ºC) and by using different devices or straws (reviewed in Arav, 2014) that allow embryo vitrification in minimal volumes; and 2) the high viscosity of vitrification media, which depends on the concentration of the CPAs. The high concentration of cryoprotectants needed to achieve high viscosity is the main concern of vitrification because it can be toxic for the embryo and may cause osmotic damage (Libermann *et al*., 2002). The cooling rate and the concentrations of CPAs are inversely related. The faster cooling is undertaken, the lower CPAs concentration is necessary to achieve vitrification (Liu *et al*., 2012). This is a key point to reduce toxicity of vitrification solutions.

Important aspects of successful cryopreservation protocols: the classics and the new trends

New embryo cryopreservation devices

Slow freezing is commonly performed using the traditional 0.25 ml straws. However, the vitrification containers have been considerably developed throughout the last years, and new devices have been developed (Reviewed in Arav, 2014) that minimize the volume in which embryos are vitrified (0.1-1 µl). These vitrification containers, which increase the cooling and warming rates (up to 20000ºC/min), have been classified into groups: 1) surface techniques and open systems, which permit the highest cooling rate and also high warming rates by direct exposure to solutions; and 2) close systems, which allow high cooling rates with the advantage of being safer and easier to handle. The main limitation of these devices is the expensive cost and the low number of embryos that they can hold, which is a major drawback for the routinely embryo cryopreservation in polytocous animals such as pigs. To overcome this problem, systems that allow the cryopreservation of large number of embryos such as the hollow fiber (Matsunari *et al*., 2012) or the easily available method of paper container (Kim *et al*., 2012) have come up lately. Furthermore, new trends aim to automate sample preparation for mammalian embryo vitrification using digital microfluidic devices (Pyne *et al*., 2014).

Cryopreservation media and cryoprotectants

Freezing and vitrification media are aqueous cryoprotectants solutions based in either phosphate-buffered or Hepes-buffered culture media. Although some cryopreservation and warming media are commercially available for cattle, equine and pigs, most of the reported data regarding embryo cryopreservation in livestock animals have been obtained using “handmade” solutions. Commonly these media contain serum or serum components. Nowadays, there is a growing concern regarding the convenience of using chemically defined media for embryo cryopreservation, which will eliminate sanitary risk and will reduce sources of variation among laboratories. In this sense, synthetic media have been already described for bovine (Hasler, 2010) and pig embryo vitrification (Sánchez-Osorio *et al*., 2010). The commercialization of these synthetic media will be of great benefit for the widespread application of this technology.

Slow freezing and vitrification require the exposure of embryos to CPAs in order to prevent the formation of ice crystal. During the last decades a wide range of cryoprotectants has been used in embryo cryopreservation protocols, which can be divided in three groups: 1) Low molecular weight permeating CPAs such as glycerol, Dymethylsulfoxide (DMSO),

propylene glycol, ethylene glycol (EG) and other alcohols; 2) Low molecular weight non-permeating CPAs which include sucrose, trehalose and other sugars; and, 3) High molecular weight non-permeating CPAs such as polyvinyl alcohol and other polymers (reviewed in Palasz and Mapletoft, 1996).

In practice, slow freezing protocols commonly implicate the use of a single permeating CPA, whereas vitrification involves the use of mixtures of two permeating CPAs in combination with a sugar (reviewed by Palasz and Mapletoft, 1996; Kasai and Mukaida, 2004). Because vitrification requires high CPAs concentrations, toxicity is an important issue to consider when improving vitrification protocols. Such high CPA concentration can lead to chemical toxicity and osmotic injury. In this way, combinations of two permeable CPAs have been successfully used to reduce the toxicity of individual agents during the vitrification of embryos of several mammalian species (Ishimori *et al*., 1992; Vicente *et al*., 1994). Furthermore, the addition of a non-permeating CPA such as sugar or a macromolecule to the vitrification solution significantly reduces the amount of permeating CPA required for vitrification and therefore decreases the toxicity (Liebermann *et al*., 2002, Liu *et al*., 2012). The most common and accepted CPA for vitrification is EG, which has low toxicity and is highly permeable (Emiliani *et al*., 2000). Each cryoprotectant has a different permeability and thus, a balance between concentration of cryoprotectant, exposure time and working temperature needs to be determined to allow successful vitrification. Adjustments to the protocols depending on the target species or the embryonic developmental stage are also required. Thus, while DMSO, EG and sucrose is the preferred mixture for pig and bovine embryo cryopreservation, glycerol and EG seems to be the usual cryoprotectants chosen for equine embryos (Barfield *et al*., 2009; Kingma *et al*., 2011).

Slow freezing is worldwide used for *in vivo*-derived bovine, ovine and goat embryos, resulting in appropriate farrowing rates (Table 1). Vitrification has not replaced slow freezing so far, but is an alternative for situations where traditional cryopreservation procedures yield unsatisfactory results. This is the case of porcine embryos, early developmental stages and *in vitro* produced embryos (reviewed in Vajta, 2000).

Table 1. Summary of farrowing rates published after transfer of cryopreserved in vivo-derived embryos in different livestock species.

Species	Reference	Cryopreservation procedure	ET Method	Number of ETs	Number of Embryos/ recipient	Farrowing rate (%)
Bovine	Reviewed in Hasler, 2014	Slow freezing	Surgical	586	1-2	71
		Slow freezing	Non-surgical	72		60
	Van Wagtendonk-de Leeuw,1997	Vitrification	Non-surgical	393	1	44.5
		Slow freezing		335		45.1
Pig	Martinez *et al*., 2015	SOPS-Vitrification	Non-surgical	33	40	72.7
	Gomis *et al*., 2012	SOPS-Vitrification	Non-surgical	10	35	50
	Cuello *et al*., 2005	OPS-Vitrification	Non-surgical	21	20	42.9
Sheep	Bettencourt *et al*., 2009	OPS-Vitrification	Laparoscopy	11	2	54.6
		Slow freezing		19	2	68.4
	Green *et al*., 2009	OPS-vitrification	Laparoscopy	44	1	55.8
		Slow-freezing	Laparoscopy	43	1	38.6
	Papadopoulos *et al*., 2002	OPS-Vitrification	Surgical	10	2	50
	Baril *et al*., 2001	0.25 ml straws-Vitrification	Surgical	25	2	72
Goat	Guignot *et al*., 2006	Slow freezing	Surgical	26	2	69
		0.25 ml straws-Vitrification		29	2	48
		OPS-Vitrification		37	2	22
Equine	Hinrichs, 2012	DM Vitrification method	Non-surgical	8	1	75**
	Choi *et al*., 2011	DM Vitrification method	Non-surgical	7	1	71**
	Eldridge-Panuska *et al*., 2005	0.25 ml straws-Vitrification	Non-surgical	26	1	62*

ET: Embryo transfer; *Day 20 of pregnancy approximately; **Heart beat stage. OPS: Open Pulled Straw; SOPS: Superfine OPS; DM: Fine-diameter microloader pipette tips using dimethylsulfoxide.

Thawing and warming protocols

Unlike slow freezing and vitrification procedures, thawing and warming protocols are very similar, using both of them very high heat rates. Typically, embryo containers are removed from liquid nitrogen and embryos are placed in solutions with decreasing concentrations of sucrose (or other non-permeating sugar) that make the CPAs to exit the embryos by producing an osmotic gradient. Today, the development of efficient direct thawing and warming methods that allow the direct transfer of embryos without removing of CPAs is essential for the use of cryopreserved embryos under field conditions. Direct transfer protocols were first described for bovine *in vivo* derived frozen embryos (Leibo, 1984). Since then, these procedures have been widely adopted allowing the direct transfer of bovine vitrified/thawed embryos with pregnancy rates similar to the ones obtained after the transfer of fresh embryos (60%, 3/5; Saha *et al*., 1996). Currently, the main challenge in this species is to develop a direct transfer method for *in vitro* produced bovine embryos. Inaba *at al*., reported acceptable pregnancy rates with *in vitro* derived vitrified bovine embryos (44.4%, 4/9; Inaba *et al*., 2011). Although no data on calving rate have been reported from these researchers, the results seem promising. For other species, direct warming and transfer methods have also been developed for vitrified embryos with encouraging farrowing rates in pig (42.9%, 9/21; Cuello *et al*., 2005; and 50%, 5/10; Gomis *et al*., 2012), goat (56%, 14/25; Guignot *et al*., 2006) and sheep (57.1%, 12/21; Green *et al*., 2009). In addition, promising pregnancy rates have been achieved with vitrified equine embryos (62%, 16/26; Eldridge-Panuska *et al*., 2005).

Effect of cryopreservation on the embryo: what we see and what we don't see

Alterations at the cellular level

During cryopreservation there is a risk of fracture damage, which has a higher incidence in the slow freezing procedures (Kasai *et al*., 1996). In addition, the equilibrium step of any cryopreservation protocol can cause an osmotic shock that may result in a shrunken embryo. Osmotic injury can disrupt the cytoskeleton (Dobrinsky *et al*., 2000). Depolimerization of microtubules and microfilaments have been observed after cryopreservation and traditional embryo vitrification using 0.25 ml straws (reviewed in Dobrinsky, 1997). To avoid this damage, cytoskeletal stabilizer agents such as Cytochalasin B has been proposed during the vitrification process in pigs. However, when ultra-rapid vitrification procedures were used to cryopreserved porcine morulae and blastocysts, cytoskeletal stabilizers were not necessary (Tharasanit *et al*., 2005; Cuello *et al*., 2010).

Slow freezing (Fair *et al*., 2001; Dalcin *et al*., 2013) and vitrification (Fabian *et al*., 2005; Cuello *et al*., 2007a; Dalcin *et al*., 2013; Chrenek *et al*., 2014) cause ultrastructural changes in embryos such as accumulation of cellular debris, an increase of vesicles and changes in the trophoblastic microvilli. Cryopreservation also induces abnormal distribution of mitochondria (Nagai *et al*., 2006), mitochondria swelling, alteration in the mitochondria shape and the rupture of their membranes (Cuello *et al*., 2007a). Recently, Dalcin *et al*. (2013) reported not only morphological alterations but also disturbed mitochondrial activity in frozen and vitrified embryos. Since mitochondria are essential for aerobic metabolism and ATP production in the embryo, the addition of glycine as a protector of mitochondria to the vitrification media has been proposed in order to overcome the above mentioned detrimental effects (Zander-Fox *et al*., 2013).

Despite all these morphological changes at a cellular level, if they are slight, the embryo is able to regenerate and eliminate death cells. In this case, the normal morphology of the embryo can be almost entirely restored after 24 h of culture without affecting the embryo viability (Vajta *et al*., 1997).

Alterations at the molecular level

Slow freezing and vitrification affect the DNA integrity (Cuello *et al*., 2005, Fabian *et al*., 2005, Kader *et al*., 2009). Considering that increased DNA fragmentation in cryopreserved embryos is partly caused by a surplus of free radicals, the addition of antioxidants to media could reduce this effect (Hosseini *et al*., 2009). Cryopreserved embryos have also shown altered expression of a number of genes when compared to fresh embryos (Mamo *et al*., 2006; Stinshoff *et al*., 2011; Shaw *et al*., 2012). Most of these alterations are related to homeostasis, metabolism and regulation of cellular and physiological activities such as cell proliferation, the cell cycle, developmental, biosynthesis, respiration and stress-related gene expression (Boonkusol *et al*., 2006; Mamo *et al*., 2006; Stokes *et al*., 2007). For example, altered Heat shock protein A1 and Solute Carrier 2 A3 gene expression has been observed in frozen-thawed embryos, which are indicators of heat stress and solute carrier functions (Kuzmany *et al*., 2011; Stinshoff *et al*., 2011). It seems logical that embryos tried to compensate for the osmotic shock and cold-conditions by altering their metabolism. This adaptation and plasticity has consequences for the embryo and little is known about the potentially effect on them and the subtle effect on the offspring (Thompson *et al*., 2007). On the other hand, the endometrium, considered as ultimate sensor of quality and healthy state of the embryos, may distinguish between fresh and vitrified embryos (Almiñana *et al*., 2014), as demonstrated by the altered gene expression

of the uterus towards the frozen embryos when compared to fresh embryos. Moreover, in rabbits it has been observed that vitrification modifies the pattern of gene and proteins expression in the placenta after implantation (Saenz-de-Juanjo *et al*., 2014).

Furthermore, when metabolism alterations were monitored in regard of the pyruvate uptake in vitrified and frozen embryos, slow-frozen embryos were more metabolically impaired than those that were vitrified (Lane *et al*., 2002). Research so far implies that vitrification induces less negative alterations on the embryo proteome and energy metabolism than slow freezing (Varghese *et al*., 2009).

Taken together, the altered gene, protein and metabolic expression of cryopreserved embryos with the differently response of the endometrium to frozen and fresh embryos may explain the inferior farrowing rates obtained with cryopreserved embryos compared to fresh ones (Papadopoulos *et al*., 2002; reviewed in Hasler *et al*., 2014).

Epigenetic effects

There is an increasing concern that cryopreservation may induce epigenetic marks and long-term alterations in the embryo. To date, there are very limited and contradictory studies regarding the possible epigenetic effects of the cryopreservation process on the embryo. On one side, some research has shown that vitrification does not alter gene methylation patters in mouse blastocyst (Zhao *et al*., 2012). On the other side, vitrification has been found to increase gene methylation in bovine two-cell embryos (Zhao *et al*., 2012). Moreover, the process of embryo vitrification itself significantly augmented the loss of methylation in the *H19* differentially methylated domain in mouse foetuses derived from vitrified embryos (Wang *et al*., 2010). DNA methylation is a key epigenetic modification, which is essential for normal embryonic development. The complex DNA methylation patterns are established and maintained by DNA methyltransferases (DNMTs). Recently, Petrussa *et al.* (2014) reported that cryopreservation resulted in disturbed expression patterns of DNMTs in human preimplantation embryos. These findings call up for further research to assess whether these disturbed embryonic DNMT expression patterns may have long-term developmental consequences for the embryo.

Personal considerations

The aim of this point is to go through personal considerations and practical aspects to take into account when embryo cryopreservation is used in farm animals.

How are embryos selected for cryopreservation?

The greatest factor affecting freezability is embryo quality, a feature that is difficult to evaluate objectively. To date, morphology evaluation by stereomicroscopy is the most employed and useful tool to evaluate embryo quality (Cuello *et al*., 2007a; Dalcin *et al*., 2013). Currently, time-lapse imaging of preimplantation embryos has been suggested as a helpful tool that may allow embryologists to be more objective in scoring embryos. Time-lapse data in conjunction with traditional morphology embryo scoring may allow better selection of embryos for cryopreservation and subsequent transfer (Conaghan *et al*., 2013).

For how long can an embryo be cryopreserved?

It has been demonstrated in different species that long storage of frozen embryos has no effect on their post-thaw survival, implantation rates, clinical pregnancy, miscarriage and live birth (up to 20 years in humans: Riggs *et al*., 2010; up to 3 years in pigs: Sanchez-Osorio *et al*., 2010; after 15 years in bovine: Fang *et al*., 2014; after 7.5 years in Sheep: Yao *et al*., 2012). Storage of cryopreserved embryos for long-term periods requires temperatures below -130ºC, the glass transition temperature of water, which can be easily provided by storage in liquid nitrogen containers at -196ºC. In fact, Glenister *et al.* (1984) pointed out by using a mouse experimental model, that frozen embryos stored in liquid nitrogen will remain “alive” for at least 2000 years. In the light of these results, it seems that the major limitation for the long-term embryo storage will be the cost for the high amount of liquid nitrogen required and the storage space and equipment associate to liquid nitrogen demands. The current development of breakthrough technology that allows the storage of cells and gametes in dry state could overcome this inconvenient in a future (Arav, 2014).

Does size matter? When being big is a problem and being small is crucial

Size really matters when it comes to embryo cryopreservation. Cryopreservation of equine embryos represents a challenge related to their size (reviewed in Stout, 2012). While the use of slow freezing and vitrification methods in small equine blastocysts (<300 µm of diameter) has been effective, their use in expanded blastocysts (>300 µm of diameter) has resulted in poor outcomes after transfer (Hinrichs, 2012). Timing of the period to obtain small equine blastocysts is uncertain and it is limited to 24 h (Betteridge, 2007). This technical difficulty makes necessary to improve the cryopreservation of expanded blastocysts. The problems associated to the cryopreservation of expanded equine blastocysts seem to be related to the blastocoele size and to the presence of the embryonic capsule (reviewed in Stout, 2012; Hinrichs, 2012). Currently, the reduction of equine

embryo size by aspiration of blastocoel content with a piezo drill or laser has been proposed (Choi *et al*., 2011; Scherzer *et al*., 2011). These studies have reported promising results on embryo survival rates, suggesting that the large volume of equine embryos is the primary impediment for successful cryopreservation.

But not only the size of equine embryos is a challenge, the size of the vitrification drop is also a matter of concern. A minimal volume is one of the most important factors for the effectiveness of vitrification as mentioned above (reviewed in Arav, 2014). Thus, keeping the volume as small as possible is a "must" when using any vitrification device.

Does fat matter? The lipid composition of the embryo

In this case, the matter of concern is more related to the impact of the fat in the cryopreserved embryo. The large amount of lipid droplets in the embryo cytoplasm, which is more evident in some species like pigs, in *in vitro* produced embryos and in early developmental stages, makes the embryos more sensitive to chilling injuries. To overcome this problem, mechanical removal of lipids from the embryo prior cryopreservation was proposed in pigs (Nagashima *et al*., 1995). However, since manipulations that disrupt the zona pellucida should be avoided, partial delipidation by chemical agents has been proposed as a more adequate strategy. In this sense, Forskolin (Men *et al*., 2006; Cuello *et al*., 2013) and L-carnitine (Takahashi *et al*., 2013) have proved to increase the cryopreservation ability of early and *in vitro*-derived embryos.

Cryopreservation "à la carte"

As we have been mentioning, the efficiency of any cryopreservation protocol is affected mainly by the species, the embryo quality, the origin of the embryo (*in vivo*- or *in vitro*-derived) and the developmental stage. Each species is unique in many aspects and thus, what may work for one species, might not work for another. While satisfactory results have been obtained after transfer of cryopreserved embryos in human, mice, cattle, pig, sheep and goat, poor results have been achieved in other domestic species such as equine (Table 1). Differences in the cryopreservation ability among species have been partly ascribed to differences in the embryo lipid composition. This is the reason for the low success of slow freezing in pigs (Polge and Willadsen, 1978; Nagashima *et al*., 1994). Moreover, embryos from different species present special features that may affect the cryopreservation procedures, such as the equine embryonic capsule (Hinrichs, 2012) or the different permeability to the CPAs (Jin *et al*., 2011).

Embryo size, water, lipid content and permeability of the plasma membrane vary not only among species but also among different developmental stages in the same species, which affect the cryopreservation outcome (Agca *et al*., 1998; Sánchez-Osorio *et al*., 2008; Jin *et al*., 2011). Although most embryo cryopreservation studies in human have shown slight or no differences in post-thaw survival among embryos frozen at the pronuclear, cleavage or blastocyst stage (Salumets *et al*., 2003; Moragianni *et al*., 2010), pig studies have demonstrated that morula and blastocyst are superior for vitrification purposes than 2-4 cells embryos based on embryo survival rates (Cuello *et al*., 2007b). In the same line, Asgari *et al*., (2012) and colleagues observed that the potential of 5-8 cell stage bovine embryos to survive vitrification and develop to the blastocyst stage was significantly lower than vitrified 8-16 cells and morula stage embryos. Differences in the cryopreservation ability have been also observed depending of the embryo origin (*in vivo vs. in vitro*). The high sensitivity to chilling injury and freezing of *in vitro*-derived embryos has been associated to their higher lipid content (Romek *et al*., 2009) and their lower quality (reviewed in Rizos *et al*., 2008) compared to *in vivo*-derived embryos.

To date, the perfect embryo cryopreservation protocol has not yet been established. A preliminary screening for each species of interest and embryo characteristic (developmental stage, origin, etc) is required to select the optimal cryopreservation conditions. Thus, we propose that a cryopreservation protocol "*à la carte*" may lead us to the best results for each occasion.

Acknowledgments

We thank Professor Emilio A. Martinez for his constructive reading and advice about this manuscript. This work was supported by the EU in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreenSkills fellowship, AgreenSkills programme: under grant agreement n° 267196; the MINECO-FEDER/AGL2012-38621, Madrid (Spain) and the Fundación Séneca/GERM 04543/07, Murcia (Spain).

References

Agca Y, Monson RL, Northey DL, Mazni OA, Schaefer DM, Rutledge JJ. 1998. Transfer of fresh and cryopreserved IVP bovine embryos: normal calving, birth weight and gestation lengths. *Theriogenology*, 50:147-162.

Alminana C, Caballero I, Cuello C, Parrilla I, Gil MA, Gomis J, Angel MA, Sanchez-Osorio J, del Olmo D, Lucas X, Vazquez JM, Vazquez JL, Fazeli A, Roca J, Martinez EA. 2013. The pig uterus distinguishes between vitrified and fresh embryos. *Reprod Domest Anim*, 48(suppl.1):106. (abstract).

Arav A. 2014. Cryopreservation of oocytes and embryos. *Theriogenology*, 81:96-102.

Asgari V, Hosseini SM, Forouzanfar M, Hajian M, Nasr-Esfahani MH. 2012. Vitrification of in vitro produced bovine embryos: effect of embryonic block and developmental kinetics. *Cryobiology*, 65:278-83.

Barfield JP, McCue PM, Squires EL, Seidel GE Jr. 2009. Effect of dehydration prior to cryopreservation of large equine embryos. *Cryobiology*, 59:36-41.

Baril G, Traldi AL, Cognié Y, Leboeuf B, Beckers JF, Mermillod P. 2001. Successful direct transfer of vitrified sheep embryos. *Theriogenology*, 56:299-305.

Bettencourt EM, Bettencourt CM, Chagas e Silva J, Ferreira P, Pereira Matos C, Romão RJ, Rocha A. 2009. Fertility rates following the transfer of ovine embryos ryopreserved using three protocols. *Small Rumin Res*, 82:112-116.

Betteridge KJ. 2007. Equine embryology: an inventory of unanswered questions. *Theriogenology*, 68:9-21.

Boonkusol D, Gal AB, Bodo S, Gorhony B, Kitiyanant Y, Dinnyes A. 2006. Gene expression profiles and in vitro development following vitrification of pronuclear and 8-cell stage mouse embryos. *Mol Reprod Dev*, 73:700-708.

Choi YH, Velez IC, Riera FL, Roldán JE, Hartman DL, Bliss SB, Blanchard TL, Hayden SS, Hinrichs K. 2011. Successful cryopreservation of expanded equine blastocysts. *Theriogenology*, 76:143-52.

Chrenek P, Makarevich AV, Popelková M, Schlarmannová J, Toporcerová S, Ostró A, Zivčák J, Bosze Z. 2014. Ultrastructure of vitrified rabbit transgenic embryos. *Zygote*, 22:558-564.

Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, Baker VL, Adamson GD, Abusief ME, Gvakharia M, Loewke KE, Shen S. 2013. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. *Fertil Steril*, 100:412-419.

Cuello C, Berthelot F, Martinat-Botté F, Venturi E, Guillouet P, Vázquez JM, Roca J, Martínez EA. 2005. Piglets born after non-surgical deep intrauterine transfer of vitrified blastocysts in gilts. *Anim Reprod Sci*, 85:275-286.

Cuello C, Berthelot F, Delaleu B, Venturi E, Pastor LM, Vazquez JM, Roca J, Martinat-Botté F, Martinez EA. 2007a. The effectiveness of the stereomicroscopic evaluation of embryo quality in vitrified-warmed porcine blastocysts: an ultrastructural and cell death study. *Theriogenology*, 67:970-982.

Cuello C, Gil MA, Almiñana C, Sánchez-Osorio J, Parrilla I, Caballero I, Vázquez JM, Roca J, Rodríguez-Martínez H, Martínez EA. 2007b. Vitrification of in vitro cultured porcine two-to-four cell embryos. *Theriogenology*, 68:258-264.

Cuello C, Sanchez-Osorio J, Almiñana C, Gil MA, Parrilla I, Roca J, Vazquez JM, Martinez EA, Rodriguez-Martinez H. 2010. Superfine open pulled straws vitrification of porcine blastocysts does not require pretreatment with cytochalasin B and/or centrifugation. *Reprod Fertil Dev*, 22:808-817.

Cuello C, Gomis J, Almiñana C, Maside C, Sanchez-Osorio J, Gil MA, Sánchez A, Parrilla I, Vazquez JM, Roca J, Martinez EA. 2013. Effect of MEM vitamins and forskolin on embryo development and vitrification tolerance of in vitro-produced pig embryos. *Anim Reprod Sci*, 136:296-302.

Dalcin L, Silva RC, Paulini F, Silva BD, Neves JP, Lucci CM. 2013. Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. *Cryobiology*, 67:137-145.

Dobrinsky JR. 1997. Cryopreservation of pig embryos. *J Reprod Fertil Suppl*, 52:301-312.

Dobrinsky JR, Pursel VG, Long CR, Johnson LA. 2000. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Biol Reprod*, 62:564-70.

Eldridge-Panuska WD, Caracciolo di Brienzab V, Seidel Jr GE , Squiresa EL , Carnevalea EM. 2005. Establishment of pregnancies after serial dilution or direct transfer by vitrified equine embryos. *Theriogenology*, 63:1308-1319.

Emiliani S, Van den Bergh M, Vannin AS, Biramane J, Englert Y. 2000. Comparison of ethylene glycol, 1,2-propanediol and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. *Hum Reprod*, 15:905-910.

Fabian D, Gjorret JO, Berthelot F, Martinat-Botte´ F, Maddox-Hyttel P. 2005. Ultrastructure and cell death of in vivo derived and vitrified porcine blastocysts. *Mol Reprod Dev*, 70:155-165.

Fahy GM, MacFarlane DR, Angell CA, Meryman HT. 1984. Vitrification as an approach to cryopreservation. *Cryobiology*, 21:407-426.

Fahy, GM, Rall, WF. 2007. Vitrification: an overview. In: Liebermann J, Tucker MJ (Ed.). *Vitrification in Assisted Reproduction: a User's Manual and Troubleshooting Guide*. Boca Raton, FL: CRC Press. pp. 21-32.

Fair T, Lonergan P, Dinnyes A, Cottell DC, Hyttel P, Ward FA, Boland MP. 2001. Ultrastructure of bovine blastocysts following cryopreservation: effect of method of blastocyst production. *Mol Reprod Dev*, 58:186-189.

Fang Y, Zeng S,Fu X, Jia B, Li S, An X, Chen Y, Zhu S. 2014. Developmental competence in vitro and in vivo of bovine IVF blastocyst after 15 years of vitrification. *Cryo Lett*, 35:232-238.

Glenister PH, Whittingham DG, Lyon MF. 1984. Further studies on the effect of radiation during the storage of frozen 8-cell mouse embryos at -196 degrees C. *J Reprod Fertil*, 70:229-234.

Gomis J, Cuello C, Sanchez-Osorio J, Gil MA, Parrilla I, Algel MA, Maside C, Del Olmo D, Vazquez JM, Roca J, Martinez EA. 2012. Non-surgical deep intrauterine transfer of superfine open pulled Straw (SOPS)-vitrified porcine embryos: evaluation of critical steps of the procedure. *Theriogenology*, 78:1339-1349.

Green RE, Santos BF, Sicherle CC, Landim-

Alvarenga FC, Bicudo SD. 2009. Viability of OPS vitrified sheep embryos after direct transfer. *Reprod Domest Anim*, 44:406-410.

Guignot F, Bouttier A, Baril G, Salvetti P, Pignon P, Beckers JF, Touzé JL, Cognié J, Traldi AS, Cognié Y, Mermillod P. 2006. Improved vitrification method allowing direct transfer of goat embryos. *Theriogenology*, 66:1004-1011.

Hasler JF. 2010. Synthetic media for culture, freezing and vitrification of bovine embryos. *Reprod Fertil Dev*, 22:119-125.

Hasler JF. 2014. Forty years of embryo transfer in cattle: a review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. *Theriogenology*, 81:152-69.

Hinrichs K. 2012. Assisted reproduction techniques in the horse. *Reprod Fertil Dev*, 25:80-93.

Hosseini SM, Forouzanfar M, Hajian M, Asgari V, Abedi P, Hosseini L, Ostadhosseini S, Moulavi F, Safahani Langrroodi M, Sadeghi H, Bahramian H, Eghbalsaied Sh, Nasr-Esfahani MH. 2009. Antioxidant supplementation of culture medium during embryo development and/or after vitrification-warming; which is the most important? *J Assist Reprod Genet*, 26:355-364.

Inaba Y, Aikawa Y, Hirai T, Hashiyada Y, Yamanouchi T, Misumi K, Ohtake M, Somfai T, Kobayashi S, Saito N, Matoba S, Konishi K, Imai K. 2011. In-straw cryoprotectant dilution for bovine embryos vitrified using cryotop. *J Reprod Dev*, 57:437-443.

International Embryo Transfer Society (IETS). 2014. 2013 statistics of embryo collection and transfer in domestic farm animals. Available on: http://www.iets.org/pdf/comm_data/December2014.pdf.

Ishimori H, Takahashi Y, Kanagawa H. 1992. Viability of vitrified mouse embryos using various cryoprotectant mixture. *Theriogenology*, 37:481-487.

Jin B, Kawai Y, Hara T, Takeda S, Seki S, Nakata Y, Matsukawa K, Koshimoto C, Kasai M, Edashige K. 2011. Pathway for the movement of water and cryoprotectants in bovine oocytes and embryos. *Biol Reprod*, 85:834-847.

Kader A, Agarwal A, Abdelrazik H, Sharma RK, Ahmady A, Falcone T. 2009. Evaluation of post-thaw DNA integrity of mouse blastocysts after ultrarapid and slow freezing. *Fertil Steril*, 91:2087-2094.

Kasai M, Zhu SE, Pedro PB, Nakamura K, Sakurai T, Edashige K. 1996. Fracture damage of embryos and its prevention during vitrification and warming. *Cryobiology*, 33:459-464.

Kasai M, Mukaida T. 2004. Cryopreservation of animal and human embryos by vitrification. *Reprod BioMed Online*, 9:164-170.

Kim YM, Uhma SJ, Gupta MK, Yanga JS, Limd JG, Dasb ZC, Heod YT, Chunge HJ, Kongf IK, Kimd NH, Leeb HT, Koa DH. 2012. Successful vitrification of bovine blastocysts on paper container. *Theriogenology*, 78:1085–1093.

Kingma SE, Thibault ME, Betteridge KJ, Schlaf M, Gartley CJ, Chenier TS. 2011. Permeability of the equine embryonic capsule to ethylene glycol and glycerol in vitro. *Theriogenology*, 76:1540-1551.

Kuzmany A, Havlicek V, Wrenzycki C, Wilkening S, Brem G, Besenfelder U. 2011. Expression of mRNA, before and after freezing, in bovine blastocysts cultured under different conditions. *Theriogenology*, 75:482-494.

Lane M, Maybach JM, Gardner DK. 2002. Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Hum Reprod*, 17:2686-2693.

Leibo SP. 1984. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. *Theriogenology*, 21:767-790.

Leibo SP, Songsasen N. 2002. Cryopreservation of gametes and embryos of non-domestic species. *Theriogenology*, 57:303-326.

Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. 2002. Potential importance of vitrification in reproductive medicine. *Biol Reprod*, 67:1671-1680.

Liu J, Phy E, Yeomans E. 2012. Theoric considerations regarding slow cooling and vitrification during cryopreservation. *Theriogenology*, 78:1641-1652.

Mamo S, Bodo S, Kobolak J, Polgar Z, Tolgyesi G, Dinnyes A. 2006. Gene expression profiles of vitrified in vivo derived 8-cell stage mouse embryos detected by high density oligonucleotide microarrays. *Mol Reprod Dev*, 73:1380-1392.

Martinez EA, Martinez CA, Nohalez A, Sanchez-Osorio J, Vazquez JM, Roca J, Parrilla I, Gil MA, Cuello C. 2015. Nonsurgical deep uterine transfer of vitrified, in vivo-derived, porcine embryos is as effective as the default surgical approach. *Scient Rep*, 5:10587.

Matsunari H, Maehara M, Nakano K, Ikezawa Y, Hagiwara Y, Sasayama N, Shirasu A, Ohta H, Takahashi M, Nagashima H. 2012. Hollow fiber vitrification: a novel method for vitrifying multiple embryos in a single device. *J Reprod Dev*, 58:599-608.

Mazur P. 2004. Principles of cryobiology. *In*: Fuller BJ, Lane N, Benson EE (Ed.). *Life in the Frozen State*. Boca Raton, FL: CRC Press. pp. 3-65.

Men H, Agca Y, Riley LK, Critser JK. 2006. Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. *Theriogenology*, 66:2008-2016.

Moragianni VA, Cohen JD, Smith SE, Schinfeld JS, Somkuti SG, Lee A, Barmat LI. 2010. Outcomes of day-1, day-3, and blastocyst cryopreserved embryo transfers. *Fertil Steril*, 93:1353-1355.

Nagai S, Mabuchi T, Hirata S, Shoda T, Kasai T, Yokota S, Shitara H, Yonekawa H, Hoshi K. 2006. Correlation of abnormal mitochondrial distribution in

mouse oocytes with reduced developmental competence. *Tohoku J Exp Med*, 210:137-144.

Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Seamark RF, Nottle MB. 1994. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol Reprod*, 51:618-622.

Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Nottle MB. 1995. Cryopreservation of porcine embryos. *Nature*, 30;374(6521):416.

Palasz AT, Mapletoft RJ. 1996. Cryopreservation of mammalian embryos and oocytes: recent advances. *Biotechnol Adv*, 14:127-149.

Papadopoulos S, Rizos D, Duffy P, Wade M, Quinn K, Boland MP, Lonergan P. 2002. Embryo survival and recipient pregnancy rates after transfer of fresh or vitrified, in vivo or in vitro produced ovine blastocysts. *Anim Reprod Sci*, 74:35-44

Petrussa L, Van de Velde H, De Rycke M. 2014. Dynamic regulation of DNA methyltransferases in human oocytes and preimplantation embryos after assisted reproductive technologies. *Mol Hum Reprod*, 20:861-874.

Polge C, Willadsen SM. 1978. Freezing eggs and embryos of farm animals. *Cryobiology*, 15:370-373.

Pyne DG, Liu J, Abdelgawad M, Sun Y. 2014. Digital microfluidic processing of mammalian embryos for vitrification. *PLoS One*, 9:e108128.

Rall WF, Fahy GM. 1985. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature*, 313(6003):573-575.

Riggs R, Mayer J, Dowling-Lacey D, Chi TF, Jones E, Oehninger S. 2010. Does storage time influence postthaw survival and pregnancy outcome? An analysis of 11,768 cryopreserved human embryos. *Fertil Steril*, 93:109-115.

Rizos D, Clemente M, Bermejo-Alvarez P, De La Fuente J, Lonergan P, Gutiérrez-Adán A. 2008. Consequences of in vitro culture conditions on embryo development and quality. *Reprod Domest Anim*, 43:44-50.

Romek M, Gajda B, Krzysztofowicz E, Smorag Z. 2009. Lipid content of non-cultured and cultured pig embryo. *Reprod Domest Anim*, 44:24-32.

Saenz-de-Juanjo MD, Marco-Jimenez F, Schmaltz-Panneau B, Jimenez-Trigos E, Viudes-de-Castro MP, Peñaranda DS, Jouneau L, Lecardonnel J, Lavara R, Naturil-Alfonso C, Duranthon V, Vicente JS. 2014. Vitrification alters rabbit foetal placenta at transcriptomic and proteomic level. *Reproduction*, 147:789-801.

Saha S, Otoi T, Takagi M, Boediono A, Sumantri C, Suzuki T. 1996. Normal calves obtained after direct transfer of vitrified bovine embryos using ethylene glycol, trehalose, and polyvinylpyrrolidone. *Cryobiology*, 33:291-299.

Salumets A, Tuuri T, Mäkinen S, Vilska S, Husu L, Tainio R, Suikkari AM. 2003. Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen-thawedembryo transfer. *Hum Reprod*, 18:1890-1895.

Sanchez-Osorio J, Cuello C, Gil MA, Almiñana C, Parrilla I, Caballero I, Garcia EM, Vazquez JM, Roca J, Martinez EA. 2008. Factors affecting the success rate of porcine embryo vitrification by the Open Pulled Straw method. *Anim Reprod Sci*, 108:334-344.

Sanchez-Osorio J, Cuello C, Gil MA, Parrilla I, Maside C, Almiñana C, Lucas X, Roca J, Vazquez JM, Martinez EA. 2010. Vitrification and warming of in vivo-derived porcine embryos in a chemically defined medium. *Theriogenology*, 73:300-308.

Saragusty J, Arav A. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction* 141:1-19.

Scherzer J, Davis C, Hurley DJ. 2011. Laser-assisted vitrification of large equine embryos. *Reprod Domest Anim*, 46:1104-1106.

Shaw L, Sneddon SF, Brison DR, Kimber SJ. 2012. Comparison of gene expression in fresh and frozen-thawed human preimplantation embryos. *Reproduction*, 144:569-582.

Stinshoff H, Wilkening S, Hanstedt A, Brüning K, Wrenzycki C. 2011. Cryopreservation affects the quality of in vitro produced bovine embryos at the molecular level. *Theriogenology*, 76:1433-1441.

Stokes PJ, Hawkhead JA, Fawthrop RK, Picton HM, Sharma V, Leese HJ, Houghton FD. 2007. Metabolism of human embryos following cryopreservation: implications for the safety and selection of embryos for transfer in clinical IVF. *Hum Reprod*, 22:829-835.

Stout TA. 2012. Cryopreservation of equine embryos: current state-of-the-art. *Reprod Domest Anim*, 47:84-89.

Takahashi T, Inaba Y, Somfai T, Kaneda M, Geshi M, Nagai T, Manabe N. 2013. Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro. *Reprod Fertil Dev*, 25:589-599.

Tharasanit T, Colenbrander B, Stout TA. 2005. Effect of cryopreservation on the cellular integrity of equine embryos. *Reproduction*, 129:789-798.

Thompson JG, Mitchell M, Kind KL. 2007. Embryo culture and long-term consequences. *Reprod Fertil Dev*, 19:43-52.

Vajta G, Hyttel P, Callesen H. 1997. Morphological changes of in-vitro-produced bovine blastocysts after vitrification, in-straw direct rehydration, and culture. *Mol Reprod Dev*, 48:9-17.

Vajta G. 2000. Vitrification of the oocytes and embryos of domestic animals. *Anim Reprod Sci*, 60/61:357-364.

Vajta G. 2013. Vitrification in human and domestic animal embryology: work in progress. *Reprod Fertil Dev*, 25:719-27.

Van Wagtendonk-de Leeuw AM, Den Daas JH, Rall WF. 1997. Field trial to compare pregnancy rates of bovine embryo cryopreservation methods: vitrification and one-step dilution versus slow freezing and three-

step dilution. *Theriogenology*, 48:1071-1084.

Varghese AC, Nagy ZP, Agarwal A. 2009. Current trends, biological foundations and future prospects of oocyte and embryo cryopreservation. *Reprod Biomed Online*, 19:126-140.

Vicente JS, Garcia-Ximenez F. 1994. Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. *Theriogenology*, 42:1205-1215.

Wang Z, Xu L, He F. 2010. Embryo vitrification affects the methylation of the H19/Igf2 differentially methylated domain and the expression of H19 and Igf2. *Fertil Steril*, 93:2729-2733.

Whittingham DG, Leibo SP, Mazur P. 1972. Survival of mouse embryos frozen to -196 degrees and -269 degrees C. *Science*, 27(178): 411-414.

Yao YC, Qi MY, Lu MH, Wang SM, Li W, Han HB. 2012. Long-term cryopreservation had no adverse effect on viability of embryos and their offspring insheep. *Anim Reprod Sci*, 136:42-46.

Youngs CR, Leibo SP, Godke RA. 2011. Embryo cryopreservation in domestic mammalian livestock species. *In*: Hemming D (Ed). *Animal Science Review*. Oxfordshire, UK: CABI. pp. 245-256.

Zander-Fox D,Cashman KS, Lane M. 2013. The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development. *J Assist Reprod Genet*, 30:107-116.

Zhao XM, Du WH, Hao HS, Wang D, Qin T, Liu Y, Zhu HB. 2012. Effect of vitrification on promoter methylation and the expression of pluripotency and differentiation genes in mouse blastocysts. *Mol Reprod Dev*, 79:445-50.